Method of Identifying Genetically Modified Papaya (PRSV-YK-resistant Strains) for Seeds and Leaves

I. Points to Note

- All testing steps, including sample preparation, shall be performed in an area with low variation in ambient temperature and humidity and no air flow.
- This method involves measurement of trace amounts of organic material. After washing the grinding devices and containers and weighing devices with neutral detergent or similar, the DNA shall be completely decimated and dried (e.g.: dry heat sterilized at 180ºC for 30 minutes).
- The sample preparation place and the testing place shall be spatially separated from each other to prevent contamination.

II. Sample Grinding

(1) Seed grinding and extraction of DNA

The seeds to be tested shall be intact, not crushed, and contain no foreign matter. After confirming the absence of jelly-like coats and other adherent matter on the seed surface, wash the seeds with 1% SDS solution 10 times, then rinse with sterile water 3 times and dry at 65ºC for 2 hours. If the seeds have not been dried completely, dry them further at 65ºC.

In the present method, GM quicker 2 (Nippon Gene Co., Ltd.) is used to extract the DNA from the seeds regardless of whether the 3-seed method or the multiple-seed method is employed. However, since the amount of reagents added and other factors differ between the two methods, the operating course, from seed grinding to DNA extraction, for each method is described separately below.

① The 3-seed method
(This is a method in which the DNA is extracted from three papaya seeds at a time)

Expose three seeds to ultraviolet rays using sterile tweezers at a clean bench for 30 minutes or more, and then place in a thick sterile polyethylene bag*[1].

Grind the seeds inside the polyethylene bag using a pestle at normal temperature. To prevent the bag from being perforated while grinding, immobilize the seeds in the upper two-third area of the bag, and slowly squash them while in the bag using a pestle until the entire content flattens. After the seeds have been squashed, rub them in the bag with a pestle, the mouth of a test tube (approximately 10 mm in inside diameter), or the lid of a 50 mL centrifugal tube.

Add 800 μL of GE1 buffer solution to the ground sample, and mix (see photo). While being careful to prevent contamination, transfer the mixture to a 1.5 mL Eppendorf tube to the maximum possible extent using a pipette or similar, add 10 μL of RNase A and 20 μL of Proteinase K, mix using a vortex mixer for 30 seconds while avoiding the formation of sample lumps and then allow to stand at 65ºC for 15 minutes. Add

100 µL of GE2-K buffer solution and vortex. Centrifuge at ≥13,000 ×g, 4ºC for 10 minutes. Following this, transfer 550 µL of the supernatant to a new 1.5 mL Eppendorf tube, add 200 µL of GB3 buffer solution and 200 µL of ethanol (100%), and then mix by inverting the tube 10 to 12 times. After applying 650 µL of the mixture to a spin column, centrifuge at ≥13,000 ×g, 4ºC for 30 seconds and discard the eluate. Repeat this operation until the entire volume of the mixture has been applied. Following this, apply 650 µL of GW buffer solution, centrifuge at ≥13,000 ×g, 4ºC for 1 minute and discard the eluate. Transfer the spin column to a new 1.5 mL tube, add 50 µL of sterile water, allow the mixture to stand at room temperature for 3 minutes, centrifuge at ≥13,000 ×g for 1 minute, and use the resulting eluate as the DNA sample stock solution.

*1: For thick polyethylene bags for sample grinding (Taiyousha Co., Ltd.), product No.:6-631-01 (75 mm × 130 mm × 0.1 mm) or equivalent shall be used.

<For reference>

Place seeds in a polyethylene bag, squash using a pestle, and then grind using the side face of the lid of the centrifugal tube or similar.

Add 800 µL of GE1 buffer solution and mix.

② Multiple-seed method

(This method is capable of detecting one positive papaya seed in 100 using qualitative real-time PCR as described below. The DNA is extracted from the same single ground sample in duplicate and each DNA extract is subjected to the real-time PCR as described below.)

Place 100 seeds, along with stainless steel beads in a 50 mL Falcon tube using sterile tweezers. Grind the seeds using a Shake Master cell disruption device (Bio Medical Science Inc, Tokyo, Japan; hereinafter BMS) or similar*[2]. After removing the beads, gather the portions of sample adhering to the tube walls on the bottom using a sterile drug spoon, and vortex vigorously.

Transfer each 100 mg powder sample to a 1.5 mL Eppendorf tube. Add 800 µL of

GE1 buffer solution to each tube and mix. Add 10 μL of RNase A and 20 μL of Proteinase K to each tube and vortex for 30 seconds while avoiding the formation of sample lamps, and then allow to stand at 65ºC for 15 minutes. Add 100 μL of GE2-K buffer solution to each tube and vortex. Centrifuge at ≥13,000 ×g, 4ºC for 10 minutes. Following this, transfer 550 μL of the supernatant to a new 1.5 mL Eppendorf tube and centrifuge at ≥13,000 ×g, 4ºC for 10 minutes. Transfer the supernatant to a new 1.5 mL Eppendorf tube, add 200 μL of GB3 buffer solution and 200 μL of ethanol (100%) and then mix by inverting the tube 10 to 12 times. After applying 650 μL of the mixture to a spin column, centrifuge at ≥13,000 ×g, 4ºC for 30 seconds, and discard the eluate. Repeat this operation until the entire volume of the mixture has been applied. Following this, apply 650 μL of GW buffer solution, centrifuge at ≥13,000 ×g, 4ºC for 1 minute and discard the eluate. Transfer the spin column to a new 1.5 mL tube, add 50 μL of sterile water, allow to stand at room temperature for 3 minutes, centrifuge at ≥13,000 ×g for 1 minute, and use the resulting extract as the DNA sample stock solution.

*2: The conditions used to grind 100 papaya seeds using the Shake Master (BMS) are as follows:

(Grinding conditions:
Add one 15 mm stainless steel bead and grind the sample at 600 rpm for 2 minutes, then at 1,000 rpm for 30 seconds. Alternatively, add one 20 mm zirconia bead and one 10 mm zirconia bead, and grind the sample at 1,000 rpm for 1 minute.)

If the Shake Master (BMS) is not available, an equivalent method of grinding, such as a mortar and pestle, shall be used.

(2) Grinding of leaves and extraction of DNA

In the present method, a QIAGEN DNeasy Plant Mini Kit (QIAGEN K.K.) is used to extract the DNA from leaves. The present method is capable of detecting as little as one positive papaya leaf in 100 by qualitative real-time PCR as described below using leaf sections prepared by the procedure described in this protocol. The method can be performed provided that 50 leaf sections are available. The DNA shall be extracted in triplicate from the same single ground sample, and each DNA extract shall be subjected to real-time PCR.

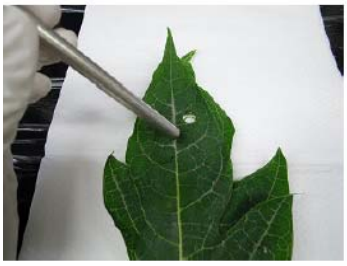
Punch out a section from each leaf using a cork borer 12.5 mm across (outside diameter). Place all leaf sections obtained in a 50 mL tube filled with sterile water and wash thoroughly using a vortex mixer. Repeat this washing operation three times.

Spread the sample uniformly over Kimtowel wiping cloth and dry in a dryer set at 65ºC for 3 hours. Grind thoroughly using a pestle or milling machine*[3] until the sample becomes powdery. Weigh out 50 mg of the ground sample in a 2 mL Eppendorf tube and add 500 μL of AP1 buffer solution pre-warmed to 65ºC. Store the remaining unconsumed sample in a frozen state at −20ºC or lower.

*3: The conditions used to grind sections from 100 papaya leaves using the Shake Master (BMS) are as follows:

Grinding conditions:
Grind the sample using one 15 mm stainless steel bead at 800 rpm for 1 minute.
Alternatively, grind the sample using three 10 mm zirconia beads at 1,000 rpm for 1 minute.

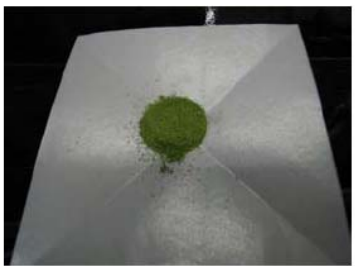
<For reference>



Punch out one section from each leaf using a cork borer. Place sections from 100 leaves in a 50 mL tube containing sterile water and wash thoroughly using a vortex mixer.



Discard the water using gauze, a drainer net, or similar method. After repeating the washing operation three times, dry the leaves at 65ºC for 3 hours.


Mill and mix the dry leaves using a mechanical mill or a mortar. Transfer the milled product to drug-wrapping paper, and weigh out 50 mg in a 2.0 mL tube

Add 5 µL of RNase A, mix vigorously by vortexing and warm at 65ºC for 15 minutes. Add 162 µL of AP2 buffer solution and vortex vigorously for 10 seconds. Allow to stand for 15 minutes on ice and then centrifuge at ≥10,000 ×g, 15ºC for 10 minutes. Apply the supernatant to a QIAshredder spin column, centrifuge at ≥10,000 ×g for 4 minutes, and then transfer the eluate to a 1.5 mL tube. Add a mixture of ethanol and AP3 buffer solution to a volume of 1.5 times that of the eluate. Apply 600 µL of the mixture to a DNeasy Mini spin column, centrifuge at ≥10,000 ×g for 1 minute, and discard the eluate. Repeat this operation until no mixture remains. Thereafter, apply 500 µL of a mixture of ethanol and AW buffer solution, centrifuge at ≥10,000 ×g for 1

minute and discard the eluate. Repeat this operation three times in total. Discard the eluate and centrifuge at ≥10,000 ×g for 20 minutes to dry the DNeasy Mini spin column. Transfer the DNeasy Mini spin column to the kit-attached centrifugal tube, add 50 μL of sterile water pre-warmed to 65ºC, allow to stand for 5 minutes, centrifuge at ≥10,000 ×g for 1 minute, and use the resulting extract as the DNA sample stock solution. Store the DNA sample stock solution in a frozen state at –20ºC or lower.

(3) Determination of DNA purity in the DNA sample stock solution and preparation and storage of DNA sample solutions

Take an appropriate amount of the DNA sample stock solution, dilute as appropriate with sterile distilled water*[4], measure the ultraviolet absorption spectrum over the wavelength range between 200 and 320 nm, and record absorbance at 260 nm and 280 nm. Following this, calculate DNA concentration assuming an absorbance of 1.0 at 260 nm to be equivalent to 50 ng/μL DNA. Also, calculate the ratio of the absorbance at 260 nm and that at 280 nm (a value between 1.7 and 2.0 indicates that the DNA is sufficiently pure; even the ratio falls outside this range, no further operations such as additional purification are required). Starting from the DNA concentration obtained, dilute the stock solution of DNA sample with sterile distilled water to 10 ng/μL and use this dilute solution as the DNA sample solution (if the DNA sample stock solution does not reach a concentration of 10 ng/μL, it shall be used undiluted). Dispense 20 μL of the DNA sample solution to microsample tubes and store in a frozen state at –20ºC or lower. The dispensed DNA sample solution should be used immediately after thawing. The remaining unconsumed solution in the container should be discarded without being stored.

*4: Use sterile distilled water as the diluent. Since different absorbance measuring apparatuses have different requirements regarding liquid volume and concentration range, the dilution rate shall be adjusted according to the apparatus used.

III. Real-time PCR

Real-time PCR shall be performed in duplicate for each DNA sample solution prepared in section II (3) above.

For detection of genetically modified papaya (PRSV-YK-resistant Strains), two kinds of primer/probe sets that detect the boundary between the cauliflower mosaic virus 35S promoter gene sequence (hereinafter referred to as CaMV 35SP) and the papaya ringspot virus coat protein (PRSV-cp) gene sequence (YK-1 and YK-2), and CaM, a primer/probe set that detects CaMV 35SP, shall be used. For the papaya positive control, a primer/probe set that detects the chymopapain (Chy) gene sequence shall be used. The base sequences of the primers/probes are shown below.

[Primers and probes for detection of genetically modified papaya (PRSV-YK-resistant Strains)]
① YK-1
YK-1F: 5’-GAT CCC CGG GTG GTC AGT-3’
YK-1R: 5’-CCG GTA TCC ACA GCT TCA TTT T-3’
YK-P: 5’-FAM- AGACGCCATGGAAGG-MGB-3’

② YK-2
YK-2F: 5’-ACA CGG GGG ACT CTA GAG-3’
YK-2R: 5’-ACC GGT ATC CAC AGC TTC-3’
YK-2P: 5’-FAM- TCC CTT CCA TGG CGT C- TAMRA-3’

③ CaM
35S-F:5’-GCC TCT GCC GAC AGT GGT-3’
35S-R:5’-AAG ACG TGG TTG GAA CGT CTT C-3’
35S-P:5’-FAM- CAA AGA TGG ACC CCC ACC CAC G-TAMRA-3’

[Primer pairs and probes for the papaya positive control]
Chy
Q-Chy-1F2: 5’-CCA TGC GAT CCT CCC A-3’
Q-Chy-2R: 5’-CAT CGT AGC CAT TGT AAC ACT AGC TAA-3’
Q-Chy-P: 5’-FAM-TTC CCT TCA T(BHQ1)CC ATT CCC ACT CTT GAG A-3’

The black-hole quencher 1 (BHQ1) of T-base shall be used as the quencher for the Q-Chy-P probe, the MGB of G-base as the quencher for the YK-P probe, and TAMRA as the quencher for the YK-2P and 35S-P probes.

(1) Real-time PCR (Applied Biosystems 7900HT, Applied Biosystems 7500)

(1)-1 Preparation of PCR reaction liquid

The PCR reaction liquid shall be prepared to a final volume of 25 μL/well. The amount of each reagent added per well shall consist of: 12.5 μL of TaqMan Gene Expression Master Mix*[5], 0.4 μL of each subject primer pair solution (concentration of each primer: 50 μmol/L), 0.25 μL of the subject probe solution (10 μmol/L), and 8.95 μL of sterile ultrapure water. Mix the required amounts of these reagents according to the number of test samples needed to yield a final PCR pre-mix solution, dispense 22.5 μL of the solution per well, and then add 2.5 μL of each DNA sample solution.

At the same time, prepare a blank PCR reaction liquid sample without adding any DNA sample solution*[6].

After completion of the operation, seal the plate from just above*[7] to ensure complete closure of the wells. This step shall be performed carefully using a dedicated sealing applicator to avoid seal wrinkles. Finally, examine the bottom of the wells for bubbles. Remove any bubbles that may be present on the bottom of the wells by gently tapping the edges of the plate. After confirming the absence of bubbles in the plate, set ABI PRISM Optical Cover Compression Pad*[8] with the brown face up on the top surface of the plate.

*5: TaqMan Gene Expression Master Mix
Since this reagent is highly viscous, caution shall be exercised to ensure complete mixing during the mixing operation. Insufficient mixing can cause the PCR operation to fail. Always be sure that the reagent has been gently stirred and centrifuged just prior to use. Use only after the solution has been spun to the bottom of the sample tube.

*6: Non-Template Control (NTC)
In the DNA sample solution adding step, add 2.5 μL of sterile distilled water in place of the DNA sample solution to each well for the NTC.
*7: 96-well plate, seals, and sealing applicator
Use the MicroAmp Optical 96-Well Reaction Plate (Life Technologies Japan Ltd.) and the ABI PRISM Optical Adhesive Cover (Life Technologies Japan Ltd.). For details regarding the sealing, refer to the manuals attached to the respective products.
*8: ABI PRISM Optical Cover Compression Pad
Use the ABI PRISM Optical Cover Compression Pad (Life Technologies Japan Ltd.).
This is not to be used with Applied Biosystems 7500.

(1)-2 Plate information settings

Prior to starting the reaction, make plate information settings. The items to be set consist of choice and positioning of sample and probe characteristics. Specifically, on a new sheet, set a sample choice (NTC: Non-Template Control, UNKN: DNA sample solution) while making sure that it corresponds to the position on the plate that has been prepared. Regarding probe characteristics, set the Reporter to the FAM mode. Set the Quencher to ① the None mode for YK-1, ② the TAMRA mode for YK-2, ③ the TAMRA mode for CaM, and ④ the Non Fluorescent mode for Chy. Set the Passive Reference to the ROX mode. For run mode settings, choose the 9600 emulation mode. Set the Sample Volume to the 25 μL mode.

(1)-3 PCR amplification

Set the plate to the PCR apparatus and start the reaction and data acquisition. The reaction conditions are as follows: Heat the sample at 50ºC for 2 minutes, then at 95ºC for 10 minutes, and start the reaction using the hot start method. Thereafter, carry out the amplification reaction for 50 cycles, each cycle comprising heat treatment steps at 95ºC for 15 seconds and at 60ºC for 1 minute. After confirming the 0-minute remaining time indication, stop the reaction and analyze the measurements.

(2) Analysis and rating of results

(2)-1 Analysis of results

For detection of both genetically modified papaya (PRSV-YK-resistant Strains) and the papaya positive control, the results obtained shall be rated by identifying an exponential amplification curve and Ct value on an amplification plot, and a clear exponential increase in fluorescence intensity from fluorescein amidite (FAM) on the multicomponent view.

For all of YK-1, YK-2, and CaM samples, if an exponential amplification curve is visually identified on the amplification plot, the sample shall be suspected of being positive for presence of genetically modified papaya (PRSV-YK-resistant Strains).

Subsequently, choose a threshold line (Th. line) that crosses the stable exponential amplification curve above the maximum noise width of the ΔRn at baseline (between 3 cycles and 15 cycles) on the amplification plot*[9]. Analyze the Th. line to determine the possibility of obtaining a Ct value therefrom. If both concurrently tested wells yield a Ct value less than 43, the result of the detection test shall be rated as positive. If only one

of the two concurrently tested wells yields a Ct value less than 43, carry out real-time PCR again and repeat this operation until an acceptable result is obtained. For any positive test as determined above, analyze the multicomponent view and confirm the following: a visible exponential increase in FAM fluorescence intensity, the absence of a clear drop in ROX fluorescence intensity and a gradual increase in FAM fluorescence intensity. For any DNA sample solution that does not yield a Ct value less than 43 in the test of the papaya positive control, perform qualitative PCR again using real-time PCR and subsequent steps. If this attempt fails to yield a Ct value less than 43, the measurement for the DNA sample solution shall be discarded.

*9: Because the ΔRn value on the amplification plot is variable depending on the status of each model, it is difficult to present specific numerical figures for universal settings of the Th. line. For this reason, a Th. line that crosses the stable exponential amplification curve above the maximum noise width of the ΔRn at baseline (between 3 cycles and 15 cycles) on the amplification plot shall be chosen. Note that for both Applied Biosystems 7900HT and Applied Biosystems 7500 models, this value appears to be in the range of 0.1 to 0.2.

(2)-2 Rating of results

The decision on whether the result is positive/negative for genetically modified papaya (PRSV-YK-resistant Strains) shall be made in accordance with the table below.

If an inconsistent result is obtained from among duplicate or triplicate DNA samples extracted from the same single ground sample, no decision shall be made and the testing shall be performed again beginning with DNA extraction.

Check the finding against the Rating/ countermeasures field in the table. For samples rated as requiring retesting, perform testing again beginning with real-time PCR. For samples that yield a retest result that does not allow a positive/negative decision for PRSV-YK-resistant Strains, further retesting shall be performed beginning with DNA extraction using a ground sample reserve that was stored by the test-performing organization. If this attempt fails to confirm a positive/negative test result, contact the test orderer (the test orderer will consider taking appropriate actions, including testing by a third-party organization using a seed reserve stored by the test-performing organization).

<Table>

Chy for detection of endogenous gene from papaya	YK-1 for detection of PRSV-YK-resistant Strains	YK-2 for detection of PRSV-YK-resistant Strains	CaM for detection of CaMV 35SP	Rating/ countermeasures
+	+	+	+	Positive
+	+	–	+	Retesting
+	–	+	+	Retesting
+	–	+	–	Retesting
+	+	–	–	Retesting
+	+	+	–	Retesting
+	–	–	+	Notification to the test orderer*10
+	–	–	–	Negative

*10: Any applicable result suggests the possible entry of a genetically modified organism other than PRSV-YK-resistant strains. The test orderer shall be notified of this fact.